Make up version of substitute specification pursuant to 37 CFR 1.125(b)(2)

IMMUNO-ADJUVANT PDT TREATMENT OF METASTATIC TUMORS

RELATED APPLICATIONS

This application claims benefit of priority from U.S. Provisional Application 60/130,519, filed April 23, 1999, which is hereby incorporated by reference as if fully set forth.

Field of the Invention

The invention relates to the use of photodynamic therapy (PDT) treatment in combination with immuno-adjuvants to treat metastatic tumors. The PDT may be conducted with any photosensitizer, but combinations comprising a benzoporphyrin derivative (BPD) are preferred for such PDT treatment.

Description of the Related Art

This invention relates to metastatic cancer. The metastatic process, which results in the growth of secondary tumors at sites distal to the primary tumor, is the cause of death in most cancers (Poste and Fidler, 1980). Although most patients with newly diagnosed solid tumors are free of detectable metastases, and about half of those patients can be cured of their disease by local cancer treatment, the remaining patients have clinically occult micrometastases that will become evident with time. Thus, at the time of primary tumor treatment, the total percentage of patients with either detectable metastases or microscopic disseminated disease is 60% (Liotta and Stetler-Stevenson, 1989).

The brain is the most favored site for metastatic spread, occurring in 25% to 30% of all cancer patients: the most frequent primary cancers, lung cancer, breast cancer and melanoma, are associated with high incidence of brain metastases (Wright and Delaney, 1989). The lung is the second most common site of metastatic spread and pulmonary metastases most frequently originate from bone and soft-tissue sarcomas (Roth, 1989).

Liver metastases commonly result from gastrointestinal tract tumors (Sugarbaker and Kemeny, 1989) and bone metastases from breast, lung and kidney primary tumors (Malawer and Delaney, 1989).

Management of a significant number of cancer cases, therefore, depends upon treating multiple tumors, traditionally through the use of surgery, radiation therapy, chemotherapy, or adjuvant therapies consisting of combinations of the three modalities.

Observations relating to tumor immunity have provided a focal point for the development of possible tumor therapy. Prehn and Main showed in 1957 that chemically induced tumors of mice were antigenic. There has been controversy concerning the relevance of chemically induced tumors, which are generally immunogenic, compared with spontaneously arising tumors in mice and human tumors which are not (Hewitt, 1979; Hewitt *et al.*, 1976).

The issue was addressed by Boon *et al.* who showed that mutagenized antigenic variants of non-immunogenic tumors could generate immunological protection in mice against the parent tumor; that is, the mutagenized and parent tumors shared antigens (Boon *et al.*, 1994). The results suggested that spontaneous experimental tumors and human tumors were antigenic and could be made immunogenic through the appropriate augmentation of the immune system (Boon *et al.*, 1994). Subsequent studies confirmed that the immune system could be made to recognize weakly immunogenic tumors by transforming tumor cells with genes for the expression of cytokines, co-stimulatory molecules, or MHC molecules (Gajewski *et al.*, 1995; Pardoll, 1993).

Also, *in vitro* culture of tumor-infiltrating lymphocytes from tumor-bearing mice and cancer patients with cytokines and irradiated tumor cells, and re-infusion of the activated lymphocytes can result in tumor regression (Burger *et al.*, 1996; Schultze *et al.*, 1997). Finally, tumor antigens recognized by the cells of the immune system have been identified in both animal models and human tumors (Jaffee and Pardoll, 1996). Tumor

antigens recognized by T lymphocytes in human melanomas are the most fully characterized set of tumor antigens and may be non-mutated, widely distributed molecules, unique and mutated proteins, or normal proteins that are overexpressed in tumors (Robbins and Kawakami, 1996).

One result from the observations concerning tumor immunity is cancer immunotherapy. For centuries it has been observed that many types of diseases, including cancer, can be improved or even cured following attacks of erysipelas, an acute skin infection. In 1909 William Coley reported several positive results following deliberate infection of cancer patients with bacteria in order to induce erysipelas. Although the contemporary theory explained tumor improvements or cures as the result of toxic products released during the bacterial infection, Coley's approach to cancer treatment may be regarded as the first instance of "biotherapy" (the original term) or cancer immunotherapy.

Immunotherapy of cancer, in which the immune system is modulated through the use of specific and non-specific tumor vaccines, bioactive molecules such as cytokines, or adoptive transfer of activated lymphocytes is one of the most appealing approaches to the treatment of metastatic cancers . The therapy is based on the concept that the patient's immunological tolerance of their cancer can be broken so that the cancer is recognized as foreign by the patient's immune system (Gore and Riches, 1996).

Another tumor treatment method is photodynamic therapy (PDT). PDT is based upon dye-sensitized photooxidation of diseased tissue and was originally developed as a treatment modality for solid tumors (Dougherty *et al.*, 1975). Singlet oxygen ($^1O_2$) is generated, without radical formation, through energy transfer processes from light-activated photosensitizer molecules in the "type II mechanism", and it is widely accepted that $^1O_2$ is responsible for the primary photodynamic effect *in vivo* (Weishaupt *et al.*, 1976). Membrane damage brought about by $^1O_2$-mediated lipid peroxidation leading to loss of cell integrity is thought to be the primary mode of cell killing by PDT (Henderson

and Dougherty, 1992), although metabolically regulated processes may also be involved in PDT-induced damage and cell death (Granville *et al.*, 1998; Tao *et al.*, 1996).

Photosensitizers are usually delivered intravenously and selective destruction of tumor tissue is based upon preferential uptake of the drug by neoplastic tissue and localized exposure of the tumor to the wavelength of light best suited to tissue penetration and photosensitizer activation. Necrosis of tumor tissue is a result of the direct effects of $^1O_2$ on tumor cells, and also from the anoxic conditions that develop in the tumor following disruption of tumor vasculature by PDT (Henderson *et al.*, 1985).

Following PDT, immune responses are initiated with the rapid induction of an inflammatory reaction (Henderson and Dougherty, 1992; Ochsner, 1997) involving the release of cytokines (Evans *et al.*, 1990; Gollnick *et al.*, 1997; Nseyo *et al.*, 1989), eicosanoids (Fingar *et al.*, 1991; Henderson and Donovan, 1989), and clotting factors (Fingar *et al.*, 1990; Foster *et al.*, 1991), and progresses to the activation of immune cells (Qin *et al.*, 1993; Yamamoto *et al.*, 1992; Yamamoto *et al.*, 1994) and infiltration of immune cells into PDT-treated tissue (Korbelik *et al.*, 1996). For example, tumor cells pre-treated with PDT *in vitro* were sensitised to macrophage-mediated lysis (Korbelik *et al.* 1994) and at low photosensitizer levels, PDT activated macrophage phagocytic activity (Yamamoto *et al.* 1994). Photofrin®-based PDT stimulated the release of the immunomodulatory molecules prostaglandin-E2 (Henderson *et al.* 1989) and tumour necrosis factor-α (TNF-α) (Evans *et al.* 1990) from murine macrophages. Photofrin® and light treatment induced the expression of interleukin (IL) IL-6 in HeLa cells (Kick *et al.* 1995) and within mouse tumours (Gollnick *et al.* 1997). A massive and rapid influx of granulocytes and macrophages has been described for murine tumours treated with Photofrin® and light [Golnick *et al.* 1997; Korbelik 1996; Krosl *et al.* 1995)]. PDT has been described as inducing tumor immunity (Korbelik 1996) which may be augmented by the localised administration of an adjuvant at the time of photo-irradiation (Korbelik *et*

al. 1998). Moreover, granulocyte-macrophage colony stimulating factor (GM-CSF) administered in three doses at two-day intervals, commencing 48 hours before light-irradiation, improved the curative effect of Photofrin® and verteporfin-mediated PDT against mouse tumours (Krosl *et al.* 1996).

PDT has also been shown to enhance both phagocytosis and tumor cytotoxicity when normal mouse peritoneal macrophages were treated *in vitro* (Yamamoto *et al.*, 1992; Yamamoto *et al.*, 1994) and similar treatments caused the secretion of tumor necrosis factor (TNF) (Evans *et al.*, 1990). In the clinical setting, treating bladder cancer with PDT resulted in detectable levels of interleukin (IL-1) and TNF-α in the urine of patients within 3 hours of treatment and IL-2 within 24 h in a profile that resembled treatment of bladder cancer with *Bacille Calmette Guérin* (BCG). In BCG therapy, elevated cytokine levels were associated with improvement (Evans *et al.*, 1990).

The role of the host immune system in PDT-mediated tumor eradication has recently been examined by Korbelik *et al.* by comparing the response to PDT of a solid tumor grown in immunocompetent or immunodeficient mice. PDT cured all normal mice; however, using the same treatment protocol with nude mice (which have a congenital absence of the thymus, resulting in reduced numbers of T cells but normal levels of B and NK cells) or scid mice (which are unable to complete V(D)J recombinations during T and B cell development and have no mature T and B cells), the initial tumor ablation following PDT was followed by regrowth of all of the tumors. Transferring splenic T cells to scid mice or reconstituting lethally irradiated scid mice with normal mouse bone marrow prior to PDT resulted in delayed regrowth or tumor cure (Korbelik *et al.*, 1996).

The same group observed a 200-fold increase in the number of tumor-associated neutrophils within minutes of sub-optimal photodynamic treatment and a drop in neutrophil content to near control levels at 2 hours after light treatment (Krosl *et al.*,

1995). Infiltrating mast cell numbers also increased within 5 min of light treatment and the higher level of mast cells was maintained for 4 hours after PDT. The numbers of mast cells were, however, several logs lower than the numbers of neutrophils. Approximately 10% of the total number of cells in the tumor at 2 hours after PDT were characterized as monocytes that had invaded the tumor from the circulation.

Also, there was a large population (20% of total cells) of tumor-associated macrophages in untreated tumors. Resident macrophages were equally sensitive to PDT killing as malignant cells but following PDT, tumor associated macrophages were shown to be almost 5 times more cytotoxic against tumor target cells *in vitro*, compared with macrophages isolated from untreated tumors.

Another means of stimulating the host immune response is by the use of adjuvants. Any material that increases the immune response towards an antigen is referred to as an adjuvant (see Appendix A) and while they have been used for at least 70 years in the production of traditional vaccines designed to prevent infectious diseases, adjuvants are also being developed for use in cancer vaccines. Adjuvants are able to augment immune responses through several mechanisms including: 1) causing depot formation at the site of inoculation; 2) acting as delivery vehicles which may target antigens to cells of the immune system; 3) acting as immune system stimulators.

Many of the adjuvant preparations function via several of these mechanisms. The ideal adjuvant would have safe local and systemic reactions (which would preclude general toxicity, autoimmune and hypersensitivity reactions, and carcinogenicity) be chemically defined so consistent manufacture is possible, would enhance protective (or in the case of cancer vaccines, therapeutic) immunity towards weak antigens, and would be biodegradable (Audibert and Lise, 1993; Cox and Coulter, 1997; Gupta and Siber, 1995).

The prototypical adjuvant, which is also the most potent, is Freund's Complete Adjuvant (CFA) developed in 1937 by Jules Freund. CFA consists of a preparation of killed *Mycobacterium tuberculosis* dispersed in mineral oil. When emulsified with water

soluble antigens, the vaccine stimulates both humoral (antibody-mediated) and cell-mediated immunity towards the antigens. The use of this adjuvant may result in serious side effects including organ injury via granuloma formation and autoimmune disease, and its use is restricted even in experimental animals. Incomplete Freund's Adjuvant (IFA), which lacks the mycobacterial component of CFA, is less toxic but does not enhance cell-mediated immunity. Nonetheless, IFA is currently undergoing clinical trials in cancer vaccine formulations (for example NCI-T97-0110, NCI-98-C-0142, NCI-H98-0010, NCI-T96-0033).

New adjuvants, such as the ~~Ribi Adjuvant System~~ RIBI ADJUVANT SYSTEM™ (RAS) have been designed to substitute highly purified bacterial components for *M. tuberculosis* in order to maintain the immune stimulatory properties of CFA without the side effects. A variation of RAS, ~~Detox~~ DETOX™ adjuvant, is currently in clinical trials as a component of cancer vaccines (NCI-V98-1489, NCI-96-C-0139). Others, such as Hunter's ~~TiterMax~~ TITERMAX™, which is has not been approved for clinical use but has been extensively characterized in animal systems, use completely synthetic compounds.

There have been previous attempts to combine immuno-adjuvants and PDT. Myers *et al.* injected formalin killed bacteria, *Corynebacterium parvum*, intralesionally in experimental tumors 24 hours prior to PDT in the first reported case of immuno-adjuvant PDT. The therapy improved the efficacy of hematoporphyrin derivative (Hpd)-sensitized PDT as measured by reduction in tumor volume and prolongation of survival (Myers *et al.*, 1989).

Using intralesional BCG, Cho *et al.* followed a similar protocol as Myers *et al.* to use PDT on a murine transitional cell carcinoma model (Cho *et al.*, 1992).

Korbelik's group reported results using immuno-adjuvant PDT in 1993 (Korbelik *et al.*, 1993). Initially, the group administered the immunostimulant schizophyllan (SPG), a glucan derived from *Schizophyllum communae*, in a series of intramuscular

injections into the hind leg of mice bearing a squamous cell carcinoma solid tumor grown intradermally over the sacral region of the back. Photofrin-based PDT was administered either 48 hours after the last SPG treatment or 24 hours before the first SPG injection. SPG therapy before PDT enhanced the effect of PDT on tumor cure whereas immunotherapy after PDT had no effect (Krosl and Korbelik, 1994).

Another study found that administering the macrophage activating factor vitamin D_3 binding protein macrophage activating factor (DBPMAF) intraperitoneally and peritumorally in a series starting immediately following Photofrin-sensitized PDT enhanced the PDT effect on tumor cures (Korbelik *et al.*, 1997). Later, the group examined the use of BCG and a purified and deproteinized preparation of the mycobacterium cell wall extract (MCWE) that is distributed by Bioniche Inc. (London, Ont. Can.) as Regressin, combined with PDT sensitized with Photofrin, Verteporfin, zinc(II)-phthalocyanine (ZnPC), and *meta*tetrahydroxyphenyl-chlorin (mThPC). A single injection of either MCWE or BCG directly beneath the tumor mass and immediately following PDT resulted in enhanced tumor cure rates (Korbelik and Cecic, 1998).

Nordquist et al. (U.S. Patent 5,747,475) disclose that the treatment of primary tumors in a rat model with indocyanine green (ICG) as chromophore and glycated chitosan as an immuno-adjuvant in photothermal therapy. This treatment resulted in some instances of reducing both primary and metastatic tumors as well as some instances of preventing the occurrence of metastatic tumors (see Figures 1 and 2 for effects against primary tumors; Figure 4 for effects against metastatic tumors; and Figure 5 for prevention of metastatic tumors).

Chen *et al.* combined glycated chitosan gel (GCG) prepared from crabshell chitin, with indocyanine green (ICG), injected ICG-GCG intratumorally and activated the ICG with thermal laser illumination in a rat metastatic tumor model. The treatment resulted in: a) no tumor response followed by death at 30 days post-treatment; b) reduced tumor

burden and extended survival times to 45 days; and c) reduced tumor burden but continued growth of the treated tumor, followed by reduction of both the treated primary and untreated metastasis. Some of the animals in the last group were cured of their tumors and rejected a subsequent challenge with the same tumor cells, indicating that the animals had developed tumor immunity and immunological memory (Chen *et al.*, 1997).

In the above instances, the processes were directed toward discrete or defined, localized tumors. Also, both Nordquist et al. and Chen et al. utilized photothermal mediated cell destruction as opposed to the photochemical mediated PDT discussed below, which does not cause any appreciable heating of the target tissue. Thus experimental combinations of immuno-adjuvants and PDT were attempted with little predictability as to actual efficacy and general application. Even the patent by Nordquist et al. only discloses the results from limited application of this concept with a single combination of one immuno-adjuvant (glycated chitosan) and one chromophore (ICG).

Given that the immune system plays an essential role in tumor destruction and the cytotoxic action of PDT, the present invention relates to a new therapeutic regime combining immunotherapy and PDT for the treatment and prevention of metastatic cancer.

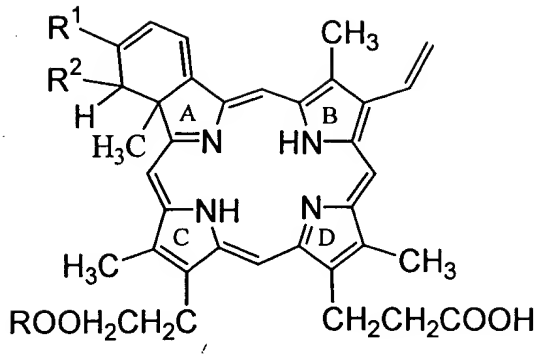
Summary of the Invention

The invention is directed to the use of photodynamic therapy (PDT) in combination with immuno-adjuvants to treat, prevent, or inhibit the development of any tumor, especially metastatic tumors. In particular, photodynamic methods employing a photosensitizer, such as a benzoporphyrin derivative (BPD), a green porphyrin, are used in combination with an immuno-adjuvant against metastatic cancer after diagnosis. Additional applications of the combination are after any primary treatment method against a diagnosed tumor to prevent the onset of as yet undetected dissemination of metastatic tumors or to treat such tumors after their appearance. The instant methods

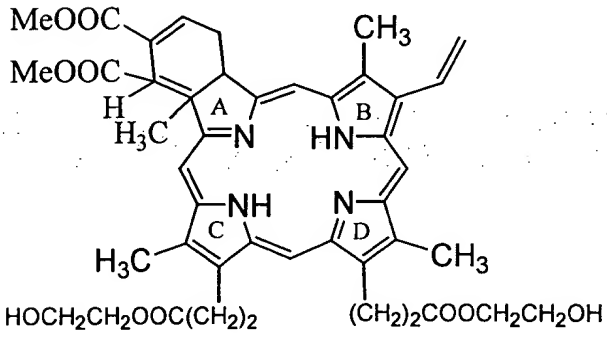
offer the benefit of efficacy against non-localized metastatic tumors either before or after their detection.

Accordingly, in one aspect, the invention is directed to a method to treat metastatic tumors, which method comprises administering to a subject with such tumors an effective amount of a photosensitizer, such as a BPD, in combination with an immuno-adjuvant and irradiating the subject with light absorbed by the photosensitizer. Such methods may be employed against metastatic tumors upon initial diagnosis of cancer in a subject or against metastatic tumors that arise after previous tumor or cancer therapy in the subject.

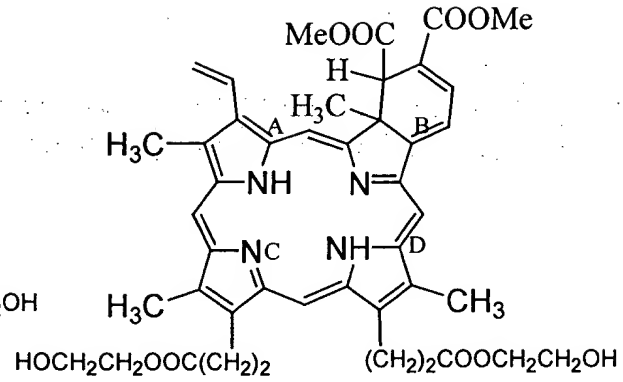
In another aspect, the invention is directed to a method to prevent or inhibit the development of metastatic tumors by the steps of administering to a subject previously having undergone cancer or tumor therapy, an effective amount of a photosensitizer, such as a BPD, in combination with an immuno-adjuvant and irradiating the subject with light absorbed by the photosensitizer. Such methods are employed even before the detection of metastasis and as such prevent, or reduce the occurrence of, metastatic tumors. The methods of the present invention specifically are contemplated for the administration of BPDs, such as those selected from the group consisting of BPD-DA, BPD-DB, BPD-MA (including BPD-MA-A also known as verteporfin) and BPD-MB (where BPDs are as presented in U.S. Patent 5,171,749, which is hereby incorporated by reference as if fully set forth) as well as the derivatives of these compounds. Particularly preferred BPDs include BPD-MA, EA6 (including A-EA6, also known as QLT 0074) and B3, where EA6 (as set forth in U.S. Patent 5,929,105, which is hereby incorporated by reference as if fully set forth) and B3 (as set forth in U.S. Patent 5,990,149, which is hereby incorporated by reference as if fully set forth) have the following structures.



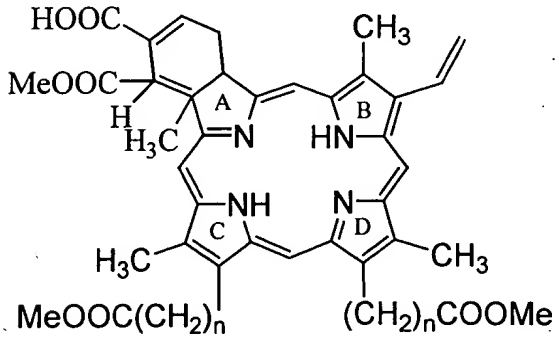
BPD-MA



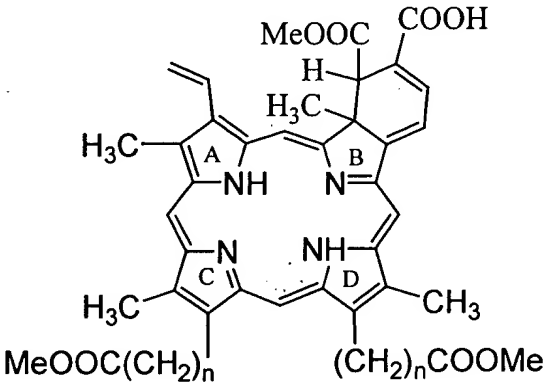
A-EA6



B-EA6



A-B3



B-B3

The methods of the present invention may be practiced with any immuno-adjuvant or combination of immunoadjuvants, including those set forth in Appendix A. Particularly preferred immuno-adjuvants are those of microbial or crustacean (chitosan) derived products. These include the ~~Ribi Adjuvant System~~ RIBI ADJUVANT SYSTEM™, ~~Detox~~™ DETOX, glycated chitosan, and ~~TiterMax~~™ TITERMAX. The ~~Ribi Adjuvant System~~ RIBI ADJUVANT SYSTEM™ and its components are described in issued US Patents 4,436,727 and 4,866,034. Preferably, the immuno-adjuvant comprises a mycobacterial cell wall skeleton component (described in US patent 4,436,727) and a component derived from lipid A of a bacterial lipopolysaccharide. Most preferably, the lipid A component is de-3-O-acylated monophosphoryl lipid A (described in US Patent 4,912,094. Additional adjuvants for use with the present invention include CFA, BCG, chitosan, and IFA. Delivery of the immuno-adjuvant may be systemic or localized.

Regarding compositions, the present invention includes pharmaceutical compositions to treat or prevent or inhibit the development of metastatic tumors, such compositions containing an amount of a photosensitizer in combination with an immuno-adjuvant effective to treat, prevent or inhibit development of metastatic tumors when administered to a subject followed by irradiation with light absorbed by the photosensitizer, and a pharmaceutically acceptable carrier or excipient. Compositions individually containing the photosensitizer and immuno-adjuvant for use together as needed are also encompassed.

Brief Description of the Drawings

The present invention will be more clearly understood by referring to the following drawings, in which:

Figure 1 shows biopsies containing experimental metastases in lungs of animals treated with immuno-adjuvant PDT, PDT only, and untreated controls.

Figure 2 shows in vitro lymphocyte proliferation in the presence of tumor antigens. See Example 4 below. The lymph nodes of mice bearing the Lewis Lung

Carcinoma (LLC) cells were removed 7-10 days following treatment with PDT or PDV. Single cell suspensions of lymphocytes were cultured in the presence of LLC and accessory cells and incubated for 5 days after which proliferation was assessed using MTS.

Detailed Description of the Invention

The present invention is directed to a procedure in which immuno-adjuvant photodynamic therapy (PDT) targets tumors, especially metastatic tumors, in some instances even before they are detectable. Thus the invention may be applied against metastatic tumors including, but not limited to, those that originate and/or result in melanoma, lung cancer, breast cancer, colon cancer, and prostate cancer. The invention may also be used in cases of lymphoid tumors that form masses. For treating metastatic tumors that have been newly diagnosed, this treatment may be utilized as a primary therapy against the tumors. For preventing or inhibiting the development of metastatic tumors, this treatment may be used as additional or follow-up therapy after primary therapy against a diagnosed tumor.

Thus following identification of metastatic tumors in a subject, an appropriate photosensitizing compound, preferably BPD-MA, EA6 or B3, will be administered to the subject in combination with an immuno-adjuvant. The order of administration of photosensitizer and immuno-adjuvant may vary, with light irradiation following administration of the photosensitizer. The immuno-adjuvant may be administered immediately after light irradiation. Simultaneous activation of the immune system by the immuno-adjuvant and PDT mediated damage to tumor cells, or initiation of immune reactions, may increase the effectiveness of treatment.

After administration, the photosensitizer will localize in tumor cells for photoactivation while the immuno-adjuvant proceeds to activate/potentiate the immune response. Light of appropriate frequency and intensity will be applied using an

appropriate light source, thereby activating the photosensitizer to destroy tumor cells and initiate immune responses, possibly by the rapid induction of an inflammatory reaction.

The formulations and methods of the present invention generally relate to administering a photosensitizer, including pro-drugs such as 5-aminolevulinic acid, porphyrins and porphyrin derivatives e.g. chlorins, bacteriochlorins, isobacteriochlorins phthalocyanine and naphthalocyanines and other tetra- and poly-macrocyclic compounds, and related compounds (e.g. pyropheophorbides) and metal complexes (such as, but not limited by, tin, aluminum, zinc, lutetium) to a subject undergoing the immuno-adjuvant PDT. Examples of photosensitizers useful in the invention include, but are not limited to, the green porphyrins disclosed in a series of patents including US Patents 5,283,255, 4,920,143, 4,883,790, 5,095,030, and 5,171,749; and green porphyrin derivatives, discussed in US Patents 5,880,145 and 5,990,149, all of which are hereby incorporated by reference as if fully set forth.

Green porphyrins are in the class of compounds called benzoporphyrin derivatives (BPD). A BPD is a synthetic chlorin-like porphyrin with various structural analogues, as shown in U.S. Patent 5,171,749. Preferably, the BPD is a benzoporphyrin derivative di-acid or mono-acid ring A (BPD-DA or BPD-MA, also known as verteporfin), which absorbs light at about 692 nm wavelength with improved tissue penetration properties.

BPD-MA, for example, is lipophilic, a potent photosensitizer, and it also appears to be phototoxic to neovascular tissues, tumors and remnant lens epithelial cells. Because of its pharmokinetics, BPD-MA may be the best candidate for use in the instant invention, but other BPDs such as EA6 and B3 or other derivatives may be used instead. Other photosensitizers, such as phthalocyanines, could be used in high concentrations sufficient to offset their relatively slower uptake. An optimal BPD for immuno-adjuvant PDT treatment or prevention of metastatic tumors should be rapidly taken up by tumor cells and should be capable of initiating an immune response upon irradiation with light to act in concert with the immuno-adjuvant.

Other non-limiting examples of photosensitizers which may be useful in the invention are photosensitizing Diels-Alder porphyries derivatives, described in US Patent 5,308,608; porphyrin-like compounds, described in US Patents 5,405,957, 5,512675, and 5,726,304; bacteriochlorophyll-A derivatives described in US Patents 5,171,741 and 5,173,504; chlorins, isobacteriochlorins and bacteriochlorins, as described in US Patent 5,831,088; meso-monoiodo-substituted and meso substituted tripyrrane, described in US Patent 5,831,088; polypyrrolic macrocycles from meso-substituted tripyrrane compounds, described in US Patents 5,703,230, 5,883,246, and 5,919,923; and ethylene glycol esters, described in US Patent 5,929,105. All of the patents cited in this paragraph are hereby incorporated by reference as if fully set forth. Generally any hydrophobic or hydrophilic photosensitizers, which absorb in the ultra-violet, visible and infra-red spectroscopic ranges would be useful for practicing this invention.

The preferred compounds of the present invention are the photosensitive compounds including naturally occurring or synthetic porphyrins, pyrroles, chlorins, tetrahydrochlorins, pyropheophorphides, purpurins, porphycenes, phenothiaziniums, pheophorbides, bacteriochlorins, isobacteriochlorins, phthalocyanines, napthalocyanines, and expanded pyrrole-based macrocyclic systems such as, sapphyrins and texaphyrins, and derivatives thereof. Other photosensitizers for use in the present invention are described in Redmond et al., Photochemistry and Photobiology, 70(4):391-475 (1999), which is hereby incorporated by reference in its entirety as if fully set forth. Preferably, the photosensitizer is not Photofrin™ (porfimer sodium).

A particularly preferred formulation according to the present invention will satisfy the following general criteria. First, an immuno-adjuvant capable of activating or potentiating the immune response is utilized. Second, a photosensitizer capable of rapid entry into the target tumor cells is used. Third, irradiation with light results in cytotoxicity to target tumor cells. This then results in the generation of immune responses. These criteria do not necessarily reflect a temporal sequence of events.

In one embodiment, the methods of the invention are used against metastatic tumors after initial diagnosis. In another embodiment, the methods of the invention follow removal or eradication of a solid tumor by conventional treatments such as surgery, radiation, chemotherapy or PDT, including immuno-adjuvant PDT. The latter embodiment may be used to prevent or inhibit the development of, metastatic tumors.

In practice of the invention, the immuno-adjuvant may be administered systemically or locally. Moreover, the immuno-adjuvant may be administered before, after or simultaneous with the photosensitizing BPD. This permits the adjuvant-mediated activation/potentiation of immune responses to overlap with PDT mediated damage to tumor cells and any PDT induced immune responses.

After administration of the photosensitizer, sufficient time is permitted to elapse for the compound to be taken up by the tumor cells. This time for uptake may be varied according to various parameters, including but not limited to the photosensitizer administered, the route of administration, the physiology of the subject and of the tumor cells, and the artisan's skill and experience. With green porphyrins, for example, the elapsed time may be from less than about one minute to more than about three hours, preferably from about one minute to about three hours, and more preferably from about 10 to about 60 minutes. The cells, or tissue containing them, then are irradiated at the wavelength of maximum absorbence of the photosensitizer. In the case of BPDs, the wavelength is usually between about 550 and 695 nm, as discussed above. In particular, red light is advantageous because of its relatively lower energy and the resulting lack of toxicity it poses to normal tissue while the tumor cells are destroyed.

The compositions and methods of the present invention provide a useful immuno-adjuvant PDT treatment to treat, prevent or inhibit the development of metastatic tumors. The following describes the compositions and formulations of the present invention and their clinical application. Experimental data also are presented and described.

Since adjuvants may exert their activity by stimulating other agents that potentiate the development of an immune response, another aspect of the invention is the use of such agents in combination with PDT. These agents include those that are immunomodulatory in activity and include several cytokines. Examples of cytokines for use in the present invention are IL-12 and IL-18 (where "IL" refers to interleukin), granulocyte-macrophage colony stimulating factor (GM-CSF), and interferon-γ (IFN-γ), which may be administered locally, systemically, or via expression vectors in combination with PDT.

Another approach of the invention is to utilize a cytokine in combination with a factor that acts to promote the growth of hematopoietic progenitors in the presence of a cytokine. FLT3-ligand, isolated and cloned via the corresponding FLT3 receptor [see refs. Rosnet *et al.* 1991; Matthews *et al.* 1991; Rasko *et al.* 1995; Lyman *et al.* 1993; Lyman *et al.* 1994] is an example of such a factor. Alone, FLT3-ligand has relatively little activity but in combination acts synergistically with other cytokines including IL-3, IL-6, IL-7, IL-11, IL-12 and colony stimulating factors to promote the growth of hematopoietic progenitors *in vitro* (Jacobsen *et al.* 1995). Following the repeated administration of recombinant FLT3-ligand to mice, splenomegaly, hepatomegaly as well as substantial increases in spleen and blood myeloid progenitor activity were observed (Brasel *et al.* 1996) indicating that FLT3-ligand mediates a mobilisation and expansion of hematopoietic stem cells.

Unexpectedly, mice given multiple FLT3-ligand injections displayed dramatic increases in numbers of functionally mature dendritic cells (DC) in multiple organs (Maraskovsky *et al.* 1996; Shurin *et al.* 1997; Steptoe *et al.* 1997). Bone marrow-derived DC are potent APC that perform a sentinel role for the immune system. These cells are normally present at low numbers within most tissues. Their abundant expression of major histocompatibility complex (MHC) gene products, adhesion and co-stimulatory

molecules is a receptor repertoire that serves in the productive activation of naïve and resting T lymphocytes (Steinman 1991; Banchereau *et al.* 1998). In association with T cells, DC may interact with and activate B cells and thereby regulate the formation of humoral immunity (Banchereau *et al.* 1998). DC are significant sources of interleukin-12 (IL-12), a pro-inflammatory cytokine that strongly promotes the formation of cellular immunity (Steinman 1991; Banchereau *et al.* 1998). In the generation of immune responses, DC are many times more effective than other APC types (B cells, macrophages) (Steinman 1991; Banchereau *et al.* 1998). Relatively few DC are required for the activation of large numbers of T cells. In most tissues, DC are present in an undifferentiated state, inefficient at stimulating T cells. However, these DC are highly efficient at capturing antigen and the signals provided by antigen acquisition promotes a maturation process that yields DC that are highly effective at activating T lymphocytes. DC phagocytose cells dying by apoptosis (programmed cell death), but not by necrosis (unregulated cell death), and can stimulate the expansion of numbers of antigen-specific cytotoxic T cells that recognize antigens contained within apoptotic cells (Morse *et al.* 1998; DiNicola *et al.* 1998). In contrast, macrophages are incapable of processing apoptotic cells for the formation of specific cytotoxic T cell immunity (Morse *et al.* 1998; DiNicola *et al.* 1998). The capacity of DC to instigate *de novo* immune responses has lead to their designation as "*nature's adjuvant*" (Steinman 1991; Banchereau *et al.* 1998; Young, *et al.* 1996; Schuler *et al.* 1997). Treatments that increase DC numbers and/or promote DC activation may ultimately foster specific T cell immunity.

Recent studies indicate that DC can provoke effective anti-tumour immunity in a variety of experimental systems. In mice, effective immunity against solid tumours has been induced by pre-exposure of DC *ex vivo* to tumour-derived peptides (Zitvogel *et al.* 1996), crude cell extracts from non-immunogenic tumours (Flamand *et al.* 1994), tumour cell-derived mRNA (Ashley *et al.* 1997; Boczkowski *et al.* 1996), recombinant viral

vectors (Song *et al.* 1997; Specht *et al.* 1997) or with DC-tumour cell fusions (Gong *et al.* 1997). Further, it has been demonstrated that DC can stimulate cytotoxic T cell activity against leukemic cells and lymphoma (Choudhury *et al.* 1997; Choudhury *et al.* 1999; Fujii *et al.* 1999; Hsu *et al.* 1996). DC exposed to tumour lysates or tumour-associated peptides *in vitro* had a vaccinating effect in human melanoma patients (Nestle *et al.* 1998). The formation of specific cytotoxic (CD8+) T cell reactivity appears critical for effective anti-tumour immunity (Schuler *et al.* 1997; Morse *et al.* 1998; DiNicola *et al.* 1998).

In cancer, various factors may blunt the development of anti-tumour immunity. This situation may arise from:

1) The action of soluble factors released by tumour cells that functionally impair immune cells.

2) Low or deficient expression of MHC or co-stimulatory molecules by tumour cells.

3) A low capacity of tumour cells to present tumour-specific antigens to T cells.

4) The loss of tumour-related antigens by tumour cell types.

5) Tumour cell expression of receptors (*e.g.* Fas ligand) that compromise immune cell survival.

DC are a unique immune cell population that is likely derived from a myeloid linage precursor cell. DC differentiation from bone marrow precursors is driven by the cytokines GM-CSF and TNF-α (Bancheereau *et al.* 1998). Additional cytokines including IL-4 and c-kit ligand regulate the differentiation and maturation of DC at different developmental stages (Bancheereau *et al.* 1998). After multiple FLT3-ligand injections, elevated DC numbers were found in immune and non-immune tissues including the spleen, peripheral blood, thymus, liver, lungs, peritoneal cavity, mesenteric

lymph nodes and Peyer's patches. These increases in DC numbers were approximately 17-fold in the spleen, 6-fold in the blood and 4-fold in peripheral lymph nodes. Importantly, these FLT3-ligand induced DC were as effective as splenic DC isolated from untreated mice in the induction of antigen-specific T cell responses. FLT3-ligand also modestly increased the number of natural killer (NK) cells in various regions (Shaw *et al.* 1998) and promoted the activation of NK *in vivo* by enhancing the interactions between DC and NK cells (Fernandez *et al.* 1999).

FLT3-ligand treated mice implanted with syngeneic fibrosarcoma tumour cells, exhibited either no development of the tumour or a significantly lower tumour size (Lynch 1998). *In vitro*, FLT3-ligand had no direct effect upon tumour cell growth (Lynch 1998). FLT3-ligand produces a therapeutic effect against non-immunogenic tumours (Fernandez *et al.* 1999), murine melanoma (Esche *et al.* 1998), murine lymphoma (Esche *et al.* 1998) and limited the spread of metastases to the liver (Peron *et al.* 1998). The increased availability of DC in tumour-bearing FLT3-ligand-treated subjects may foster the recognition of tumour-associated structures by DC. The interaction of DC with NK cells may simulate NK cell-mediated tumour cell lysis releasing apoptotic or necrotic cell bodies that are taken up, transported, processed and presented by DC to T lymphocytes (Fernandez *et al.* 1999).

Thus the present invention includes the use of combined PDT/FLT3-ligand anti-cancer therapy. FLT3-ligand is currently available from Immunex (Seattle, Washington) as MOBIST™, while recombinant human and mouse FLT3-ligand is available commercially from the biological reagent supplier R&D (Minneapolis, Minnesota). Based on mouse studies, FLT3-ligand may be adminstered to effect an increase in peripheral DC numbers. This may be accomplished by a regimen of regular administrations, such as a number of days for higher animals (e.g. humans). Standard PDT could be administered via intravenous injection of a photosensitiser followed later at

a pre-determined time with light irradiation. FLT3-ligand administration may be continued for a number of days after PDT.

FLT3-ligand should be administered in a manner that when PDT is applied there is a high availability of DC within the body. When the delivery of PDT is co-ordinated with an FLT3-ligand-induced zenith in DC numbers, the interaction of DC with dying tumour cells would be optimal. This circumstance would provide the patient's immune system the greatest opportunity to generate a specific and effective response to tumour antigens - potentially providing the potential to limit residual and metastatic cancer through immunologic mechanisms.

Yet another aspect of the invention involves a more direct use of dendritic cell (DC) therapy in combination with PDT. Since tumour cells may lack the capacity to directly stimulate T cell responses due to a lack of the appropriate repertoire of accessory structures (MHC, co-stimulatory molecules, etc.) for instigating the responses, the acquisition of tumour cell material by DC could lead to the formation of specific anti-tumour immunity. Thus the use of *ex vivo* culture systems may circumvent immunosuppressive influences exerted by the tumour and permit the immune sensitisation to tumour antigens.

One means of conducting this approach begins with a subject's peripheral blood DC being prepared and cultured in vitro for 24-48 hours with inactivated (optionally by PDT) tumor cells, tumor antigens, and/or any other tumor specific or related factor. These DC, as antigen presenting cells, are re-introduced into the subject, with PDT applied to the subject either before or after the re-introduction.

The Photosensitizers

The BPDs and green porphyrins useful in the method of the invention are described in detail in Levy et al., U.S. Patent No. 5,171,749 issued 15 December 1992, which is incorporated herein by reference. "Green porphyrins" refer to porphyrin

derivatives obtained by reacting a porphyrin nucleus with an alkyne in a Diels-Alder type reaction to obtain a monohydrobenzoporphyrin. Typically, green porphyrins are selected from a group of porphyrin derivatives obtained by Diels-Alder reactions of acetylene derivatives with protoporphyrin under conditions that promote reaction at only one of the two available conjugated, nonaromatic diene structures present in the protoporphyrin-IX ring system (rings A and B).

Several structures of typical green porphyrins are shown in the above cited patent, which also provides details for the production of the compounds.

Dimeric forms of the green porphyrin and dimeric or multimeric forms of green porphyrin/porphyrin combinations can be used. The dimers and oligomeric compounds of the invention can be prepared using reactions analogous to those for dimerization and oligomerization of porphyrins *per se*. The green porphyrins or green porphyrin/porphyrin linkages can be made directly, or porphyrins may be coupled, followed by a Diels-Alder reaction of either or both terminal porphyrins to convert them to the corresponding green porphyrins.

Additionally, the green porphyrin compounds used in the invention may be conjugated to various ligands to facilitate targeting to target tumor cells. These ligands include those that are receptor-specific, or immunoglobulins as well as fragments thereof. Preferred ligands include antibodies in general and monoclonal antibodies, as well as immunologically reactive fragments of both.

The green porphyrin compounds of the invention may be administered as a single compound, preferably BPD-MA, or as a mixture of various green porphyrins. Suitable formulations include those appropriate for administration of therapeutic compounds *in vivo*. Additionally, other components may be incorporated into such formulations. These include, for example, visible dyes or various enzymes to facilitate the access of a photosensitizing compound to target tumor cells.

Formulations

The photosensitizers and immuno-adjuvants of the invention may be formulated into a variety of compositions. These include liposomes, nanoparticles, and pluronic (Poloxamer) containing formulations. These compositions may also comprise further components, such as conventional delivery vehicles and excipients including isotonising agents, pH regulators, solvents, solubilizers, dyes, gelling agents and thickeners and buffers and combinations thereof. Appropriate formulations and dosages for the administration of immuno-adjuvants are known in the art. Suitable excipients for use with photosensitizers and immuno-adjuvants include water, saline, dextrose, glycerol and the like.

Typically, the photosensitizing agent is formulated by mixing it, at an appropriate temperature, e.g., at ambient temperatures, and at appropriate pHs, and the desired degree of purity, with one or more physiologically acceptable carriers, *i.e.*, carriers that are nontoxic at the dosages and concentrations employed. Generally, the pH of the formulation depends mainly on the particular use, and concentration of photosensitizer, but preferably ranges anywhere from about 3 to about 8. Preferably, the photosensitizer is maintained at a pH in the physiological range (*e.g.*, about 6.5 to about 7.5). The presence of salts is not necessary, and, therefore the formulation preferably is not an electrolyte solution. Appropriate nonantigenic ingredients, such as human serum albumin, may optionally be added in amounts that do not interfere with the photosensitizing agent being taken up by lens epithelial cells.

The particular concentration of a given BPD should be adjusted according to its photosensitizing potency. For example, BPD-DA can be used but at about a five-fold higher concentration than that of BPD-MA. Moreover, the BPD may be solubilized in a different manner than by formulation in liposomes. For example, stocks of BPD-MA or

any other BPD may be diluted in DMSO (dimethylsulfoxide), polyethylene glycol or any other solvent acceptable for use in the treatment of tumors.

Normally, the adjustment of pH is not required when liposomal BPD-MA is used, as both components have a neutral pH. However, when other solvents than liposomes are used, the pH may require adjustment before mixing the BPD with the other material. Since antioxidants may interfere with the treatment, they should generally should be avoided.

Preparation of dry formulations that are reconstituted immediately before use also are contemplated. The preparation of dry or lyophilized formulations of the compositions of the present invention can also be effected in a known manner, conveniently from the solutions of the invention. The dry formulations of this invention are also storable. By conventional techniques, a solution can be evaporated to dryness under mild conditions, especially after the addition of solvents for azeotropic removal of water, typically a mixture of toluene and ethanol. The residue is thereafter conveniently dried, *e.g.* for some hours in a drying oven.

Suitable isotonising agents are preferably nonionic isotonising agents such as urea, glycerol, sorbitol, mannitol, aminoethanol or propylene glycol as well as ionic isotonising agents such as sodium chloride. The solutions of this invention will contain the isotonising agent, if present, in an amount sufficient to bring about the formation of an approximately isotonic solution. The expression "an approximately isotonic solution" will be taken to mean in this context a solution that has an osmolarity of about 300 milliosmol (mOsm), conveniently 300 + 10 % mOsm. It should be borne in mind that all components of the solution contribute to the osmolarity. The nonionic isotonising agent, if present, is added in customary amounts, i.e., preferably in amounts of about 1 to about 3.5 percent by weight, preferably in amounts of about 1.5 to 3 percent by weight.

Solubilizers such as Cremophor types, preferably Cremophor RH 40, or Tween types or other customary solubilisers, may be added to the solutions of the invention in standard amounts.

A further preferred embodiment of the invention relates to a solution comprising a BPD compound, and a partially etherified cyclodextrin, the ether substituents of which are hydroxyethyl, hydroxypropyl or dihydroxypropyl groups, a nonionic isotonising agent, a buffer and an optional solvent. However, appropriate cyclodextrins should be of a size and conformation appropriate for use with the photosensitizing agents disclosed herein.

Summaries of pharmaceutical compositions suitable for use with the instant photosensitizers and immuno-adjuvants are known in the art and are found, for instance, in Remington's Pharmaceutical Sciences.

Administration of Photosensitizers and Immuno-Adjuvants

As noted above, the treatment of the present invention is carried out in tissues either maligned with metatstatic tumors or susceptible to their occurrence, in an afflicted subject. The photosensitizer and immuno-adjuvant containing preparations of the present invention may be administered systemically or locally and may be used alone or as components of mixtures. Preferred routes of administration are intravenous, subcutaneous, intramuscular, or intraperitoneal injections of the photosensitizers and immuno-adjuvants in conventional or convenient forms. Injection of the adjuvant into a tumor, whether primary or resulting from metastasis, is preferred. Intravenous delivery of photosensitizers is preferred, and intratumor injection may also be used when desired, as in pigmented tumor situations where the dose of PDT would be increased, for example. Oral administration of suitable oral formulations may also be appropriate in those instances where the photosensitizer may be readily administered to the tumor or tumor-prone tissue via this route.

The invention also includes the use of repeat treatments as deemed necessary by a suitable clinician or skilled worker in the field. Preferably, the treatment is repeated from 1 to about 10 times at intervals of about 1 to about 2 weeks. More preferably, the treatment is repeated from 1 to about 5 times, or most preferably for a total of 3 times, at approximately 2 week intervals.

Additionally, if the treatment is to be localized to an area of metastatic tumors suitable for topical formulations, the photosensitizers may be topically administered using standard topical compositions including lotions, suspensions or pastes.

The dose of photosensitizers and immuno-adjuvants can be optimized by the skilled artisan depending on factors such as, but not limited to, the physical delivery system in which it is carried, the individual subject, and the judgment of the skilled practitioner. It should be noted that the various parameters used for effective PDT in the invention are interrelated. Therefore, the dose should also be adjusted with respect to other parameters, for example, fluence, irradiance, duration of the light used in PDT, and time interval between administration of the dose and the therapeutic irradiation. One means of rapidly evaluating parameters for PDT/adjuvant administration is set forth below in Example 4. All of these parameters should be adjusted to produce significant damage to metastatic tumor cells and initiate an immune response without causing significant damage to the surrounding tissue. With photosensitizers, for example, the form of administration, such as in liposomes or when coupled to a target-specific ligand, such as an antibody or an immunologically active fragment thereof, is one factor considered by a skilled artisan.

Depending on the specificity of the preparation, smaller or larger doses of photosensitizers may be needed. For compositions which are highly specific to the target tumors, such as those with the photosensitizer conjugated to a highly specific monoclonal antibody preparation or specific receptor ligand, dosages in the range of 0.05-1 mg/kg are suggested. For compositions which are less specific to the target, larger dosages, up to 1-

10 mg/kg, may be desirable. The foregoing ranges are merely suggestive in that the number of variables with regard to an individual treatment regime is large and considerable deviation from these values may be expected. The skilled artisan is free to vary the foregoing concentrations so that the uptake and cellular destruction parameters are consistent with the therapeutic objectives disclosed above.

The time of immuno-adjuvant delivery may be before or after irradiation with light as well as before or after administration of the photosensitizer, although irradiation will occur after administration of the photosensitizer. The immuno-adjuvant may be delivered immediately after irradiation. This may be of particular relevance with immuno-adjuvants that are opaque or otherwise interfere with irradiation.

Without being bound by theory and in instances of BPDs being used as the photosensitizer, irradiation is thought to result in the interaction of BPD in its triplet state with oxygen and other compounds to form reactive intermediates, such as singlet oxygen, which can cause disruption of cellular structures. Possible cellular targets include the cell membrane, mitochondria, lysosomal membranes.

Each photosensitizer requires activation with an appropriate wavelength of light. With BPDs, an appropriate light source, preferably a laser or laser diode, in the range of about 550 to about 695 nm, is used to destroy target cells. An appropriate and preferred wavelength for such a laser would be 690± 12.5 nm at half maximum. Generally, cell destruction occurs within 60 seconds, and likely is sufficiently complete within about 15 to about 30 seconds. The light dose administered during the PDT treatment contemplated herein can vary, but preferably ranges between about 10 to about 150 J/cm^2. The range between about 50-100 J/cm^2 is preferred. Increasing irradiance may decrease the exposure times.

Localized delivery of light is preferred, and delivery localized to the tumor is more preferred. Delivery of light prior to photosensitizer activating light is also contemplated to improve penetration of the activating light. For example, irradiation of

pigmented melanomas with infrared light before visible red light bleaches the melanin to improve penetration of the red light.

The time of light irradiation after administration of the green porphyrin may be important as one way of maximizing the selectivity of the treatment, thus minimizing damage to structures other than the target tumor cells. Light treatment within about 3 hours before or after application of the photosensitizer should generally be attempted. Alternatively, light treatment may be simultaneous, or nearly simultaneous, with said application.

The following examples are intended to illustrate but not to limit the invention.

Example 1

Sample Animals and Tumor Model

Male, C57BL/6 mice were obtained from Charles River Canada (Montreal, QC) at 6 to 8 weeks of age. The B16-F0 and B16-F1 melanoma cell lines were obtained from the American Type Tissue Collection (Manassas, Virginia) and grown as cell cultures in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Sigma). The cells adhered to tissue culture plates, were removed for passage with 0.25% trypsin with 1.0 mM ethylenediaminetetraacetic acid (EDTA) (Gibco), and were cryo-preserved in liquid nitrogen in DMEM plus 40% FBS and 10% DMSO. Mice were injected with 5 X 10^5 tumor cells in a total volume of 50 μL subcutaneously into the shaved, right flank. The tumor size was monitored daily by measuring the diameter with vernier calipers and were treated when the tumors reached approximately 5 mm in diameter. In initial experiments, the B16-F0 and B16-F1 were characterized with respect to *in vivo* growth rates and metastatic potential and were found to be identical. Subsequently the B16-F1 cell line was used for all experiments.

Example 2

Sample Immuno-Adjuvant PDT

PDT treatment of mice bearing the B16-F1 tumor was performed as previously described for the M1 rhabdomyosarcoma mouse tumor (Richter *et al.*, 1987; Richter *et al.*, 1988; Richter *et al.*, 1991). Each mouse was weighed, warmed under infrared light for less than 5 min to dilate the blood vessels, restrained, and injected intravenously (tail vein) with Verteporfin at a concentration of 1.0 mg/kg body weight using a 28G needle. Thirty minutes later, animals were restrained and half of the animals were injected intratumorally with 50 μL of ~~Titermax~~ TITERMAX™ adjuvant (Sigma) prepared as an emulsion with sterile phosphate buffered saline (PBS) according to the manufacturers specifications. Animals were then exposed to a light dose of 100 J/cm^2 in a circular area encompassing the tumor of 1 cm diameter at 688 nm wavelength. The power density was 70 mW/cm^2 and resulted in treatment times of 24 min per animal. Following treatment, animals were monitored daily for tumor response.

Example 3

Sample Experimental Metastases

Pulmonary metastases were generated by intravenous injection of tumor cells according to standard methods described by several groups (Chapoval *et al.*, 1998; Lin *et al.*, 1998; Volpert *et al.*, 1998; Wang *et al.*, 1998). Pulmonary metastases were initiated in each group of treated mice, as described in Example 2 above, when the tumor was considered cured. This involved multiple treatments in some of the mice and all test animals were injected intravenously with tumor cells on the same day. Following PDT or immuno-adjuvant PDT animals were monitored for tumor response and if positive, Test (PDT and immuno-adjuvant PDT) and Control (naive) animals were injected with 5 X 10^5 tumor cells in 250 μl PBS via the lateral tail vein. The animals were monitored for

tumor recurrence and general health for 14 days after which the animals were sacrificed using CO_2 inhalation and their lungs removed. Pulmonary metastases were clearly visible as black tumor colonies against the normal, pink lung tissue.

Results from the above are shown in Figure 1. The B16 melanoma tumor model is inherently difficult to treat with PDT because of the absorption of light by the black melanin pigment secreted by the tumor cells. However, 10 animals completed the entire course of the experimental procedure. Five animals received PDT alone and of those animals, 3 required repeated PDT treatments to complete the tumor cure. Five animals received immuno-adjuvant PDT and 2 required second treatments with immuno-adjuvant PDT. All of the animals that had been treated with immuno-adjuvant PDT developed between 1 and 7 lung tumors at the time of dissection. One of the animals treated with PDT alone developed 6 lung colonies but the remaining 4 animals developed between 30 and 60 lung colonies. All of the control animals developed 200 to 300 lung colonies but the density of tumor growth made accurate quantification impossible (Fig. 1).

Thus immuno-adjuvant PDT evidently augments tumor immunity that develops during tumor growth and/or following PDT. Although the above example uses pigmented tumors in an experimental metastases approach, the results indicate that the combination of an immuno-adjuvant with PDT can be used for the treatment of metastatic cancer.

Example 4

Rapid Evaluation of PDT/Adjuvant (PDV) Therapy via Lymphocyte Proliferation

In order to assess the potential usefulness of various adjuvants and treatment parameters in PDV, an *in vitro* lymphocyte proliferation assay was designed and employed in a murine tumor model. The assay measures tumour-specific lymphocyte (tumor immunity) responses from animals treated with PDT and PDT combined with

adjuvant (PDV). This permits the rapid evaluation of various PDT/adjuvant administration protocols.

Female C57Bl/6 mice are implanted subcutaneously on the shaved right flank with the Lewis Lung Carcinoma (LLC) cell line. When tumours develop to approximately 5 mm diameter animals are treated with PDT or PDV. PDT is performed by delivering 1.0 mg/kg Verteporfin® i.v. 30 min prior to illumination of 125 J/cm2 delivered at 70 mW/cm2 (treatment time = 29 min, 4 sec). Animals treated with PDV receive a single 50 µl intratumoral injection of adjuvant immediately following illumination. Animals are monitored for general health and re-growth of the tumour following therapy.

Seven to 10 days following therapy, animals are sacrificed and inguinal, axillary, cervical, and periaortic lymph nodes are aseptically removed. A single cell suspension is produced from the lymph nodes and this is cultured in half-area, 96-well tissue culture plates (Corning) in the presence of titrations of freeze/thawed tumour cells and irradiated syngeneic splenocytes depleted of erythrocytes as accessory cells. The cells are cultured in the presence of recombinant interleukin-2 (Sigma), and concanavalin A (ConA) (Sigma) is utilized as a positive control to assess the proliferative capacity of lymphocytes. Following 3 to 5 days of culture, the degree of proliferation is assessed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (Owen's reagent, MTS, from Promega), a variation of the MTT assay which produces a soluble formazan product which absorbs light at 490 nm. The degree of proliferation is calculated by comparing the means of at least triplicate test wells to the means of lymphocytes cultured without antigen or mitogen (test mean - MTS background ÷ control mean - MTS background x 100 = percent proliferation).

The assays may be performed using the commercial, experimental adjuvant, ~~Ribi Adjuvant System~~ RIBI ADJUVANT SYSTEM™ (RAS) (Corixa) or ~~Detox~~ DETOX™ B-SE (Corixa) and alum for comparison.

Of those animals treated with PDV which also responded to ConA (n=7), lymphocytes proliferated to 126 ± 19% (mean ± standard deviation) of lymphocytes without antigen (see Fig. 2). Animals treated with PDT alone proliferated to 108 ± 11%. Controls using naïve animals, tumour-bearing animals treated with adjuvant alone, and proliferation in the presence of another syngeneic tumour to test specificity have also been tested.

Example 5

Sample protocol for metastatic tumors

This protocol may be used for a variety of metastatic tumors, including metastatic melanoma.

Liposomal verteporfin is injected at a dosage of 14 mg/m2 of body surface area, which is a higher dose than for treating AMD. One to three hours later, diode laser light is applied at a rate of approximately 200mW/cm2 for a total dosage of 120-180J/cm2 to the lesion being treated. The dosage of the ~~Detox~~ DETOX™ adjuvant, which is injected into the lesion after PDT, provides in the range of 100-200$_{\mu}$g of the cell wall skeleton component, and 20-30$_{\mu}$g of the monophosphoryl lipid A component. This procedure is carried out at approximately 2 week intervals. Preferably there are 3 treatments.

All references cited hereinabove and below are hereby incorporated by reference in their entireties, whether previously specifically incorporated or not.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the invention following, in

general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

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Appendix A: Adjuvant Classification

PARTICULATE ADJUVANTS

-exist as microscopic, insoluble particles

-generally, the immunogen must be incorporated into or associated with the particle.

A. Mineral-based

-insoluble, gel-like precipitate

-mineral formulations are the only adjuvants that are considered safe and effective for use in human vaccines

i. **Aluminum hydroxide (Alhydrogel)**
Superfos chemicals
~~http://~~www.superfos.com/index.htm

a. **SBAS4**
Aluminum salt combined with monophosphoryl lipid A (MPL)
SmithKline Beecham
~~http://~~www.sb.com/index.html

ii. **Aluminum phosphate (Adju-Phos)**
Superfos chemicals
~~http://~~www.superfos.com/index.htm

ii. **Calcium phosphate**
Superfos chemicals
~~http://~~www.superfos.com/index.htm

B. Water-in-oil emulsions

-microdroplets of water, stabilized by surfactant in a continuous oil phase

i. **Freund's Complete Adjuvant (FCA)**
a mixture of a non-metabolizable oil (mineral oil), a surfactant (Arlacel A), and mycobacteria (*M. tuberculosis* or *M. butyricum* in Modified FCA)
Superfos chemicals
~~http://~~www.superfos.com/index.htm

ii. **Freund's Incomplete Adjuvant (FIA)**
has the same oil/surfactant mixture as FCA but does not contain any mycobacteria

iii. **Montanide Incomplete Seppic Adjuvant (ISA) Adjuvants**
a group of oil/surfactant based adjuvants in which different surfactants

are combined with either a non-metabolizable mineral oil, a metabolizable oil, or a mixture of the two. They are prepared for use as an emulsion with aqueous Ag solution. The surfactant for Montanide ISA 50 is mannide oleate, a major component of the surfactant in Freund's adjuvants. The surfactants of the Montanide group undergo strict quality control to guard against contamination by any substances that could cause excessive inflammation, as has been found for some lots of Arlacel A used in Freund's adjuvant. The various Montanide ISA group of adjuvants are used as water-in-oil emulsions, oil-in-water emulsions, or water-in-oil-in-water emulsions. The different adjuvants accommodate different aqueous phase/oil phase ratios, because of the variety of surfactant and oil combinations. The performance of these adjuvants is said to be similar to Incomplete Freunds Adjuvant for antibody production; however the inflammatory response is usually less.
Seppic, Paris, France

C. **Oil-in-water emulsions**
-microdroplets of squalene or squalane, stabilized with surfactants in a continuous water phase, developed for human clinical trials when combined with immunomodulators RIBI ADJUVANT SYSTEM™

i. ~~**Ribi Adjuvant System**~~ **(RAS)**
4 components: (1) monophosphoryl lipid A (MPL); (2) trehalose dimycolate (TDM); (3) cell wall skeletons (CWS); (4) ↑S. ↑*typhimurium* mitogen (STM)
Ribi ImmunoChem Research, Inc.
~~http://~~www.ribi.com~~/~~

ii. **MF59**
originally developed with N-acety-muramyl-L-alanyl-2-(1',2'-dipalmitolyl-sn-glycero-3'-phospho)ethylamide (MTP-PE) however when antibody titer was endpoint, MTP-PE was not required for adjuvant activity
Chiron Corp.
~~http://~~www.chiron.com~~/~~

iii. **SBAS4**
combination of monophosphory lipid A (MPL), QS21, and a proprietary oil in water emulsion
SmithKline Beecham
~~http://~~www.sb.com/index.html

iv. DETOX
~~Detox~~™
active ingredients include MPL® (derivative of the lipid A molecule found in gram negative bacteria) and mycobacterial cell wall skeleton
Corixa Corporation
~~http://~~www.corixa.com

v. DETOX
~~Detox~~ B-SE™ for investigational use is supplied in clear glass vials. Each vial contains: 145 micrograms CWS from M. phlei, 25 micrograms MPL from S. minnesota R595, 8.1 milligrams Squalane F, 0.38 milligrams Polysorbate 80 (USP/NF), 1.62 milligrams Soy Lecithin (NF), and 88 micrograms Sterile Water for Injection (USP)
~~Detox~~ B-SE must be stored refrigerated between 2 and 8°C
DETOX ^TM

D. Immune stimulating complexes (ISCOM)
-open, cage-like structure resulting from the interaction of Quil-A with cholesterol and phosphatidycholine, human clinical trials

E. Liposomes
-single or multilamellar bilayer membrane vesicles comprised of cholesterol and phospholipid
-the immunogen may be membrane-bound or within the intermembrane spaces

F. Nano- and microparticles
-solid particles, biocompatible and biodegradable, synthetic polymers of cyanoacrylates, polycatide coglycolide (PLG) copolymer, antigen must be formulated with particle

NON-PARTICULATE ADJUVANTS

A. Muramyl dipeptide (MDP) and derivatives: Adjuvant peptides
-*N*-acetyl muramyl-L-alanyl-D-isoglutamine is the active component of peptidoglycan extracted from *Mycobacterium*, derivatives are less toxic

i. **threonyl MDP**

ii. **murabutide,*N*-acetylglucosaminyl-MDP (GMDP)**

a. Gerbu Adjuvant
Alternative to FCA. Oil is replaced by water-soluble, aliphatic quaternary amines or bio-degradable esterquats. Mycobacterium is replaced by GMDP.
Gerbu Biotechnik GmbH, Gaiberg, Germany
C-C Biotech

16766 Espola Road
Poway, CA 92064
USA

iii. **murametide**

iv. **nor-MDP**

B. Non-ionic block copolymers
-polymers composed of a region of hydrophobic polyoxypropylene (POP) flanked by regions of hydrophilic polyoxyethylene (POE), not biodegradable

i. ~~TiterMax~~ TITERMAX™
CytRx Corporation
~~http://~~www.cytrx.com~~/~~

iv. **Syntex Adjuvant Formulation-1 (SAF-1)**
Roche Bioscience (formerly Syntex Corp., Palo Alto, CA)
~~http://~~www.roche.com/pharma/Index.htm

iv. **SAF-2**

C. Saponins
-extract of Quillaia saponaria tree, saponin is crude extract of triterpenoids

i. **Quil A**
Partially purified saponin

ii. **Spikoside**
Partially purified saponin

iii. **QS21 (Stimulon)**
Purified, defined entity
Aquila Biopharmaceuticals, Inc. (formerly Cambridge Biotech Corporation)
~~http://~~www.aquilabio.com~~/~~

iv. **ISCOPREP™ 703**
Purified, defined entity

D. Lipid A and derivatives
-disaccharide of glucosamine with two phosphate groups and five or six fatty acid chains (C_{12} to C_{16} in length)

i. **monophosphoryl lipid A (MPL)**
removal of the 1' phosphate group from lipid A gives MPL

E. Cytokines

F. Carbohydrate polymers
-polymers of mannose and β1-3 glucose
-proposed as human vaccine adjuvants either mixed with or conjugated with immunogen

-stimulate macrophages and dendritic cells

G. **Derivatized polysaccharides**
-high molecular weight sulphated dextrans proposed as human vaccine adjuvants

H. **Bacterial toxins**
-potent mucosal adjuvants in animal models